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Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection

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Mastitis, the most consequential disease in dairy cattle, costs the US dairy industry billions of dollars annually. To test the feasibility of protecting animals through genetic engineering, transgenic cows secreting lysostaphin at concentrations ranging from 0.9 to 14 mg/ml in their milk were produced. *In vitro* assays demonstrated the milk's ability to kill *Staphylococcus aureus*. Intramammary infusions of *S. aureus* were administered to three transgenic and ten nontransgenic cows. Increases in milk somatic cells, elevated body temperatures and induced acute phase proteins, each indicative of infection, were observed in all of the nontransgenic cows but in none of the transgenic animals. Protection against *S. aureus* mastitis appears to be achievable with as little as 3 mg/ml of lysostaphin in milk. Our results indicate that genetic engineering can provide a viable tool for enhancing resistance to disease and improve the well-being of livestock.

The promise of genetic engineering as a tool for enhancing livestock production characteristics has remained unfulfilled. The first published project on transgenic farm animals was aimed at improving feed efficiency and increasing lean muscle mass[1], but the focus of transgenic livestock projects quickly turned to production of compounds primarily for human medical application[2–6]. Shortly thereafter, xenotransplantation applications were explored[7]. During the past two decades, a small but persistent group of laboratories continued to work towards agricultural goals[8–12], with primary objectives of growth enhancement and nutritional enrichment of animal products. Only two projects, one in chickens[13] and another in sheep[14], have attempted to improve an animal's ability to resist disease. The work reported here is an initial step towards improving the ability of dairy cattle to resist bacterial infections that cause mastitis, an endemic disease of the mammary glands.

Mastitis is reported to be the most costly disease in animal agriculture, resulting in lost profits to the US dairy industry of ~$2 billion annually[15,16]. Economic losses associated with mastitis are both direct (loss in milk production, veterinarian's time, herdsman's time, cost of drugs and cost of discarded milk) and indirect (high culling rate, extended calving intervals and reduced milk quality)[17]. Furthermore, the consequences of mastitis go beyond economics. Mastitis also seriously affects animal well-being, and is a primary reason for culling or death of dairy cattle[18].

S. aureus currently accounts for up to 30% of clinical mastitis cases and has proven difficult to control. There is currently no effective vaccine for preventing intramammary infections caused by *S. aureus* and the cure rate for treatment of *S. aureus* mastitis with antibiotics is often less than 15%. This is attributed to incomplete penetration of antibiotics throughout the gland and the potential survival of bacteria within host cells, leading to a recurrence of disease once treatment has ended[19].

Lysostaphin (EC 3.4.24.75), known primarily as a peptidoglycan hydrolase produced naturally by *Staphylococcus simulans*, efficiently cleaves glycyl-glycine bonds in the pentaglycine inter-peptide links of the peptidoglycan found in the cell wall of staphylococci. *S. aureus* is particularly sensitive to this glycyl-glycine endopeptidase. Discovered and initially characterized in 1964 (ref. 20), lysostaphin was proposed as a systemic therapy for *S. aureus* infection[21]. More recently, its efficacy against *S. aureus*-caused mastitis was demonstrated in a mouse model[22]. We extended that finding by producing genetically engineered mice carrying a lysostaphin transgene[23]. The majority of transgenic females challenged with *S. aureus* were resistant to infection. Now, we report that transgenic cattle expressing lysostaphin in their milk are resistant to *S. aureus* intramammary infection.

RESULTS

Production of transgenic cows

Transfected fibroblasts from six Jersey fetuses, serving as nuclear donor cells, were used to generate 4,007 fused couplets[24]. Activation followed by embryo culture yielded 927 good quality blastocysts of which 650 were transferred into 330 recipient heifers and cows. GFP expression at the blastocyst stage provided conformation that every embryo transferred carried a transgene. Eight calves were born alive, five of which survived to adulthood.

Lactation parameters

No differences were detected in volume of milk produced (milk yield, $P = 0.791$) or percentage milk protein ($P = 0.857$) between

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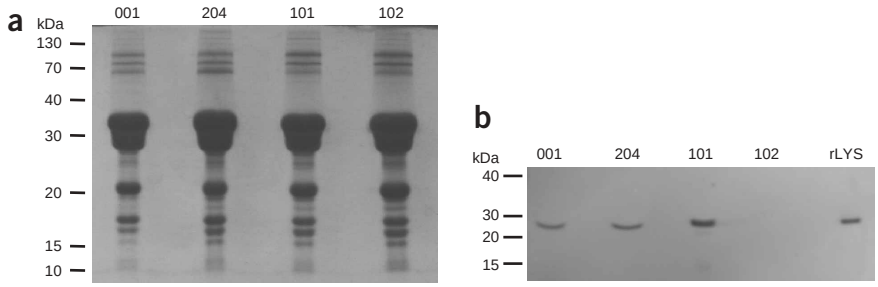

Figure 1 Protein analysis of milk from transgenic Jersey cows 001, 101 and 204 and nontransgenic Jersey cow 102. (**a**) Fifty micrograms of total proteins from transgenic cows 001, 101 and 204 and nontransgenic animal 102 were separated on a 15% polyacrylamide gel and visualized by Coomassie blue staining. (**b**) Western blot analysis of milk probed with antibody against recombinant lysostaphin: 5 µl milk from 001, 204; 1 µl of milk from 101, 102. Bacterially derived recombinant lysostaphin in saline (10 ng).

transgenics and their nontransgenic Jersey controls. Milking performance of the transgenic clones was also compared with that of the dam of the fetus from which they were produced. Neither volume ($P = 0.590$) nor percentage milk protein ($P = 0.492$) differed. Milk proteins of the transgenic animals, as visualized on a polyacrylamide gel, appeared essentially identical to those from a nontransgenic Jersey herdmate (**Fig. 1a**). However, percentage of milk fat was higher for the transgenic clones (6.7%) than for either the nontransgenic Jerseys (5.5%, $P < 0.001$) or the dam of the fetus (5.1%, $P = 0.042$) from which the nuclear donor fibroblasts were derived.

Lysostaphin concentration in transgenic cow's milk

A single protein of the predicted size was immunologically reactive to antibodies against lysostaphin. The protein was observed in the milk from three transgenic cows, but not in the milk of nontransgenic Jerseys (**Fig. 1b**). Lysostaphin concentrations, as measured by ELISA, ranged from 0.9 to 14 µg/ml in five lines of transgenic cattle (**Table 1**). Values for transgenic clones 001, 101 and 204 were determined from multiple milk samples taken during their first lactations. Milk lysostaphin concentrations for bull calf 214 and heifer calves 215 and 312 were measured in milk samples collected after induced lactations when animals were 3 to 5 months of age. All three young animals are offspring of transgenic male founders. Bull calf 214 and heifer calf 215 are offspring from the same founder (004) and had remarkably similar lysostaphin concentrations in their milk. The two heifer calves are just now reaching breeding age, so were not included in the *S. aureus* challenge study reported here. The predictive value of lactation induction in transgenic cattle has not yet been established[9]; however, several transgenic goat studies would suggest that estimates of transgene expression from an induced lactation are likely to be accurate[25,26].

Lysostaphin concentration in the milk of transgenic animals remained fairly constant during most of lactation (**Fig. 2**). Within the first three days after parturition, lysostaphin tended to be higher than during the rest of lactation. In one case (cow 101), lysostaphin increased in late lactation, as did other milk components, primarily as a result of reduced fluid volume. The transgene promoter, ovine β-lactoglobulin, was derived from a closely related species and therefore might be expected to mimic that of endogenous β-lactoglobulin. At the protein level, however, the pattern of lysostaphin concentration was not strongly associated with the concentration of bovine β-lactoglobulin (**Fig. 2**).

In vitro milk assays

Bacteriolytic activity of the milk from transgenic cows was determined in a lysis assay in which milk samples were incubated on *S. aureus* lawns (**Fig. 3**). The lytic ability of milk from transgenic cows was quantified by comparing optical densities of cleared zones with densities produced by bacterially derived recombinant lysostaphin in a dilution series. The regression model of the lysostaphin standard curve fit the data well ($r^2 = 0.98$). The *in vitro* biological activity for transgenic animals 001, 101 and 204 was equivalent to 0.08, 2.50 and 0.13 µg/ml of recombinant lysostaphin, respectively. A comparison of those values with the mass values of lysostaphin measured by ELISA indicates that transgenically produced protein is approximately 15% as active as bacterially derived recombinant lysostaphin (**Table 1**).

Because the antimicrobial activity of lysostaphin is known to extend to a number of *Staphylococcus* species[27] and its range can be broadened when combined with other antimicrobial compounds[28], it is possible that milk constituents might influence lysostaphin's activity. Therefore, milk from transgenic cow 101, the highest expressor, was tested against a variety of microorganisms. Neither 101's milk nor recombinant lysostaphin at 100 µg/ml had a discernable effect on the growth of *Escherichia coli, Klebsiella pneumonia, Pseudomonas aeruginosa, Serratia marcescens, Streptococcus agalactiae, Streptococcus dysgalactiae* or *Streptococcus uberis*. However, some inhibition of growth was observed when lawns of *Staphylococcus* species, *S. chromogenes, S. hyicus, S. epidermis, S. simulans* or *S. xylosus* were exposed to milk from cow 101 or to recombinant lysostaphin (data not shown).

S. aureus challenge study

The transgenic cows' ability to resist infection by *S. aureus* was tested by intramammary infusion of viable bacterial cultures. Of the mammary glands infused, 34 of 48 glands (71%) became infected in nontransgenic animals compared to 3 of 21 glands (14%) in transgenic animals ($P = 0.001$, **Table 2**). The highest expressing female, 101, who was infused nine times with *S. aureus*, never became infected. Transgenic cow 001, producing about 5% as much lysostaphin

Table 1 Lysostaphin concentrations and biological activity

			Assay method	
			ELISA	Lysis assay
ID (Sex)	Generation	Founder ID	Mean ± s.e.m. (µg/ml)	Mean ± s.e.m. (µg/ml)
001 (F)[a]	Founder	001	0.87 ± 0.08	0.08 ± 0.01
101 (F)[a]	Founder	101	11.03 ± 0.38	2.50 ± 0.20
204 (F)[a]	Founder	204	0.92 ± 0.03	0.13 ± 0.01
214 (M)[b]	G_1	004	5.2 ± 0.5	na
215 (F)[b]	G_1	004	5.6 ± 0.9	na
312 (F)[b]	G_1	104	13.8 ± 4.4	na

Lysostaphin concentrations were measured by ELISA and biological activity was measured by a lawn lysis assay, in the milk of three transgenic founder females and three first generation offspring of transgenic male founders.
[a]Subjects of this study. [b]Milk from induced lactations at 3–6 months of age. na, not assayed.

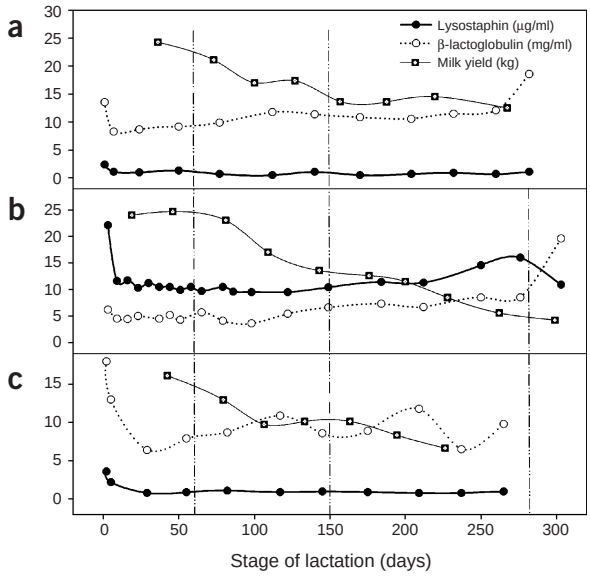

Figure 2 Milk parameters and lysostaphin concentration during the first lactation of transgenic cows. (**a**) Transgenic cow 001. (**b**) Transgenic cow 101. (**c**) Transgenic cow 204. Vertical dashed lines indicate times of *S. aureus* infusion.

as 101, was infected twice after six infusions and transgenic cow 204, expressing similarly to 001, was infected once out of six infusions. Saline-infused glands were infected once after 23 infusions. The three strains of *S. aureus,* capsular serotypes 5, 8 and 336, used during each challenge study, were similar in virulence, exhibiting infection rates of 75%, 69% and 69%, respectively.

A primary clinical indicator of mastitis is an elevation in milk somatic cells (mainly polymorphonuclear neutrophil leukocytes (PMN) and macrophages). Twenty-four hours after infusion of mammary glands with *S. aureus,* somatic cells in milk increased in nontransgenic animals but not in transgenic animals ($P = 0.003$, **Fig. 4a**). Somatic cell counts for the three transgenic animals remained relatively unchanged in the bacteria-infused glands (preinfusion = $513 \pm 84 \times 10^3$; 48 h = $601 \pm 211 \times 10^3$ cells/ml) throughout the monitoring period (168 h). This lack of response in the transgenic cows was indistinguishable from that in 23 saline-infused glands (preinfusion = $307 \pm 40 \times 10^3$; 48 h = $598 \pm 319 \times 10^3$ cells/ml, $P = 0.472$). Transgenic cow 204 had a numeric but nonstatistically significant spike in somatic cell concentration at 48 h postinfusion ($1.7 \pm 0.5 \times 10^6$ cells/ml). In contrast, somatic cell counts of *S. aureus*-infused glands of nontransgenic animals increased acutely between 24 and 30 h postinfusion to a peak approaching 30×10^6 cells/ml. The three strains of *S. aureus* elicited virtually identical somatic cell responses, both in timing of the peak response and in magnitude of response in the nontransgenic animals ($P > 0.05$). Furthermore, the stage of lactation (peak, mid- or late) at which the challenge was conducted did not influence the response in either the transgenic or nontransgenic cows ($P = 0.220$).

Elevated body temperature, indicative of a systemic immune response, increased above preinfusion levels in all ten nontransgenic cows by 24 h (**Fig. 4b**, $P = 0.045$) and returned to preinfusion levels by 48 h ($P = 0.484$). No change in body temperature was detected in the three transgenic animals during any of the challenges ($P = 0.694$).

Acute-phase blood proteins also serve as a measure of a systemic response to infection. Consistent with previous reports[29,30], the establishment of an intramammary *S. aureus* infection in nontransgenic cows increased the circulating levels of both lipopolysaccharide (LPS)-binding protein (LBP) and serum amyloid A (SAA). Elevated levels of LBP and SAA were detected in the nontransgenic animals 36 h postinfusion (**Fig. 4c,d**, respectively). In contrast, the transgenic cows had no demonstrable changes in the circulating levels of either of these acute-phase proteins throughout the study period.

DISCUSSION

The relatively low heritability of mastitis resistance hampers significant progress by selective breeding[31] and the limited efficacy of conventional disease prevention strategies also contributes to the need to investigate alternative solutions. We have chosen to explore the potential of introducing beneficial genes into dairy cattle in the hopes of reducing susceptibility to major mastitis-causing pathogens.

Transgene expression levels in the animals reported here were similar to those found in some livestock bioreactors[5], but less than in others[8,9]. The ovine β-lactoglobulin regulatory element driving this transgene has been used extensively in mice and in sheep, where it is renowned for producing α-1-antitrypsin at 35 g/l in the milk of Tracey, a bioreactor ewe[3]. The concentrations of lysostaphin in these

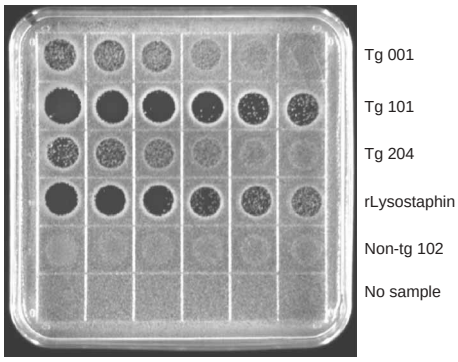

Figure 3 *S. aureus* serotype 5 lawn grown in the presence of 10-μl drops of milk from transgenic (Tg) and nontransgenic (Non-tg) Jersey cows and recombinant lysostaphin. Milk was undiluted in first column and serially diluted by half in subsequent columns. Recombinant lysostaphin was serially diluted from 1 μg/ml to 31 ng/ml. Amount of lawn clearing is proportional to staphylolytic activity in the sample.

Table 2 Infection rate of three serotypes of *S. aureus* infused into mammary glands of three transgenic and ten nontransgenic lactating cows

	S. aureus capsular polysaccharide serotype			
Cow[a]	5	8	336	Overall
Tg 001	0/2[b]	0/2	2/2	2/6
Tg 101	0/3	0/3	0/3	0/9
Tg 204	1/2	0/2	0/2	1/6
Non-tg (n =10)	12/16	11/15	11/16	34/47

During each challenge experiment each gland was infused with one of the three *S. aureus* serotypes and the fourth gland was infused with PBS. Infection was defined as *S. aureus* growth in two consecutive milk samples collected 6 to 12 h apart. None of the seven transgenic glands infused with PBS became infected. One of 16 glands infused with PBS in the nontransgenic animals became infected.

[a]001 and 204 were challenged at peak and mid-lactation, 101 was challenged at peak, mid- and late lactation. Nine nontransgenic cows were challenged at peak lactation, four at mid- and three at late lactation. Nontransgenic cows were matched by age and stage of lactation to the transgenics. [b](Number of glands infected)/(number of glands infused).

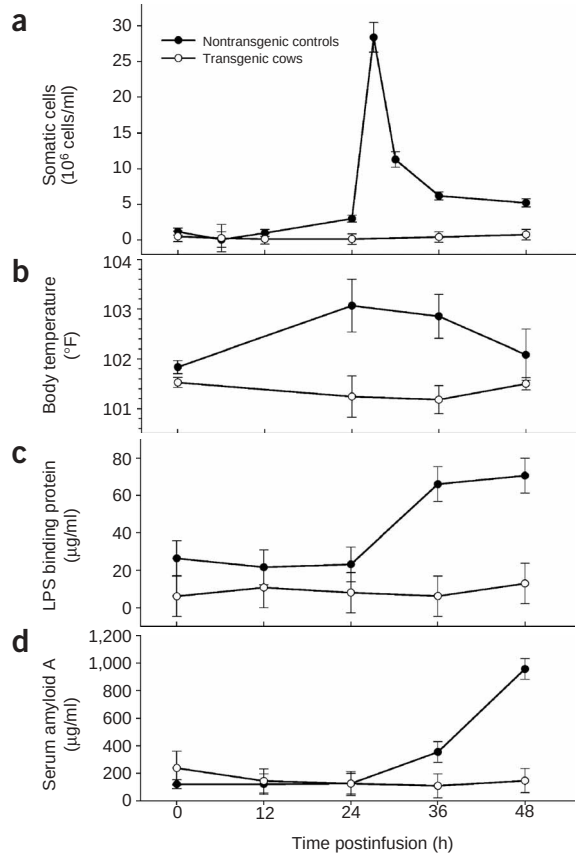

Figure 4 Response of transgenic ($n = 3$) and nontransgenic ($n = 10$) cows to intramammary infusion of 80 c.f.u. of *S. aureus* in each of three glands/cow during peak, mid- and late lactation (21 transgenic glands and 47 nontransgenic glands). (**a**) Somatic cell concentration in milk. (**b**) Body temperature. (**c**) Lipopolysaccharide (LPS)-binding protein concentration in serum. (**d**) Serum amyloid A circulating concentrations. Serum data from three transgenics and four nontransgenics in panels **c** and **d**.

cows were several orders of magnitude lower than in that exceptional sheep. Furthermore, the lysostaphin concentration of the highest expressing animal in this study (heifer 312, induced lactation) was only 1% of the highest expressor in our transgenic mouse model, where concentrations ranged from 60 μg/ml to 1.3 mg/ml[23].

Numerous explanations are possible for lower concentrations of lysostaphin in the transgenic cows, including negative consequences of transgene integration site, poor utilization of an ovine regulatory element in the bovine, or attenuated transcription rate caused by the addition of two auxiliary flanking transgenes[32,33]. Because this is, to our knowledge, the first published results for use of this promoter in cattle it is difficult to assess which, if any, of these potential explanations might account for the low expression. In a practical sense, the expression observed was adequate to test the ability of lysostaphin to reduce susceptibility to *S. aureus* infection.

The diminished bioequivalence of milk-derived lysostaphin, relative to that produced by bacteria, was expected. We have observed a reduction of similar magnitude in mice[23]. We had found that *in vitro* production of authentic lysostaphin by a mammalian cell line resulted in an inactive protein due to glycosylation. Thus, the coding sequence was modified to generate $Gln^{125,232}$-lysostaphin in which two potential glycosylation sites were removed. This prevented glycosylation of the protein, and restored partial activity. It appears that processing of the lysostaphin gene is similar in mammary glands of mice and cattle and the mechanism responsible for diminished activity is likely related to the amino acid substitutions. But we cannot exclude post-translational processing as a cause of diminished activity (**Fig. 1b**).

The innate immune system represents the first line of defense in the host response to infection and is poised to immediately recognize and respond to the earliest stages of infection. A major component of this system is the rapid induction of a set of inflammatory reactions referred to as the acute-phase response, which is characterized by fever, leukocytosis, changes in vascular permeability and metabolism, and induction of hepatically derived, acute-phase proteins[29]. In this study, 71% of *S. aureus*-challenged quarters became infected in the nontransgenic cows. Other studies using similar *S. aureus* strains observed infection rates as low as 51%[34] and as high as 90%[35]. Preparation and administration of the inoculums are likely to account for some of this variation. The infection rate between nontransgenic animals and those expressing lysostaphin leave little doubt that the transgene product conferred a protective effect. Because of the small transgenic sample size it is not possible to make any statistical inferences regarding the frequency of infection and milk lysostaphin concentration. However, the data are suggestive. The cow with the lowest *S. aureus* lytic activity had the highest infection rate (two out of six glands infected), the animal with intermediate activity had a lower infection rate (one out of six glands) and the highest expressing animal appeared to be completely protected. Furthermore, even in the infected glands, the severity and duration of the infections would likely be less than in nontransgenic animals. Actual field trials will be required to determine if the results of our challenge model are representative of typical dairy operations.

The infiltration of somatic cells into the mammary gland is a demonstration of the innate immune response to mastitis, as it is diagnostic of the disease. A significant increase in somatic cell concentration in the milk was first detected at 24 h postinfusion in nontransgenic animals. This delay in immune response is typical of an *S. aureus* infection[29]. There was no significant mean increase in somatic cells in the transgenic animals. However, one of the low expressing transgenic animals did appear to mount a relatively mild response at 48 h postinfusion. The delay and magnitude of response suggest that growth of *S. aureus* may have been retarded but not sufficiently to preclude a mild response to *S. aureus*. It would appear that the concentration of lysostaphin in the milk of the two lowest expressing animals is not adequate to completely inhibit *S. aureus*.

The lack of elevated body temperature in the transgenic animals also suggests protection by lysostaphin. Presumably, the low numbers of *S. aureus* infused were lysed almost instantly, then ingested and digested by resident macrophages and neutrophils. These cells would likely have released signaling molecules indicative of infection, but the amount released would have been much too low to cause a noticeable systemic response similar to what has been reported previously[29].

The induction and corresponding increase in the circulating levels of two acute-phase proteins, SAA and LBP, is characteristic of *S. aureus*–induced mastitis[29,30]. SAA has been shown to contribute to the inflammatory response by (i) inducing extracellular matrix-degrading enzymes, (ii) acting as a chemoattractant and (iii) inducing pro-inflammatory cytokine production[36]. LBP is a lipid transfer molecule that facilitates the recognition of Gram-negative, bacteria-derived LPS by cell surface receptors on macrophages and neutrophils[37]. In addition, LBP has been reported to facilitate immune cell

recognition of *S. aureus*–derived cell wall products, including lipoteichoic acid[38]. The lack of induction of these proteins in the transgenic animals further supports the lack of established infection in their mammary glands after the bacterial challenge.

Our group, and others, have attempted to improve farm animal well-being through genetic engineering[14,39], but this study appears to be the first to make substantial progress towards achieving the goal. Other proteins with antimicrobial activity have been expressed in the mammary glands of transgenic livestock[40,41]. However, the primary focus of those studies was either the production of nutraceuticals or food safety issues, rather than animal health. It is likely that overexpression of both lysozyme and lactoferrin confer some protection against mastitis, though there appears to be no published evidence to support that notion in a livestock species.

Milk from transgenic cow 101, the highest expressing cow, completely blocked growth of *S. aureus* in our *in vitro* lawn assay system. Her milk, diluted 8- to 16-fold, exhibited full lytic activity. Correcting for the lower mass-to-mass effectiveness of mammary gland–produced lysostaphin compared to the recombinant product, we predict that animals producing the $Gln^{125,232}$ variant of lysostaphin at concentrations of 3 μg/ml, as measured by ELISA, will be protected against *S. aureus*–caused mastitis. If that supposition is correct, and if the induced lactation accurately predicts natural lactation levels of lysostaphin, we predict that transgenic females 215 and 312 will be completely protected against *S. aureus*–caused mastitis.

As encouraging as these results are, a number of issues regarding lysostaphin will have to be resolved. We reported previously that mammary gland development in the mouse appears to be compromised by lysostaphin at expression levels 100 times higher than that observed in this study[42]. Moreover, the food safety issues presented by milk containing lysostaphin will have to be addressed in future studies. There has been some interest in lysostaphin in human medicine in the past. Although none of the clinical investigations have involved oral administration of lysostaphin, those studies do suggest that topical toxicity is not a significant issue. Lysostaphin has been shown to irritate the skin when applied topically, but the response was mild[43]. In other studies, the therapeutic benefits of lysostaphin have been tested in infants, children and adults as an intranasal mucosal treatment for *S. aureus* infections with positive effects[44,45].

Furthermore, lysostaphin's effectiveness in treating experimental endophthalmitis mediated by methicillin-resistant *S. aureus* has been demonstrated[46]. Interestingly, lysostaphin is also being explored as a food safety additive in sausage manufacture[47]. Finally, the potential for development of lysostaphin-resistant *S. aureus* does exist, which is why we are planning additional genetic engineering manipulations that we believe will diminish the practical consequences of that occurrence.

We believe the results of this study demonstrate the feasibility of using genetic engineering to introduce beneficial genes into cattle. Such transgenes could have a positive impact on the economics of the dairy industry and on animal well-being.

METHODS

Production of transgenic cattle. The transgenic Jersey cattle included in this study were produced as previously described[24]. They carry a transgene construct containing genes for neomycin resistance, green fluorescent protein and a peptidoglycan hydrolase (lysostaphin) with expression directed to mammary gland secretory epithelium. The expression of the modified lysostaphin gene[23] is regulated by an ovine β-lactoglobulin promoter (a gift from A.J. Clark, pBJ41, Roslin Institute, UK). The three transgenic heifers evaluated in this study were all cloned from the same Jersey fetus but were produced on different days from newly transfected donor fibroblasts; thus, each heifer had its transgene integrated in a different random locus. This strategy was intended to produce animals with different levels of transgenic expression. Ten unrelated nontransgenic, nonclone controls, matched by age and lactation, were also included (three Jerseys and seven Holsteins). The production, propagation and experimentation involving live animals was approved in advance by the Beltsville Animal Care and Use Committee. Production and evaluation of the animals were covered on protocols 02-027, 02-034 and 03-010.

Inoculum preparation. Frozen thawed stocks of *S. aureus*, capsular polysaccharide serotypes 5 (American Type Culture Collection (ATCC) no. 29740), 8 (ATCC 49525) and 336 (ATCC 55804)[48] were grown for 6 h at 37 °C in brain-heart infusion medium (BHI, Difco Laboratories). An aliquot was transferred to tryptic soy broth (TSB, Difco Laboratories) and incubated at 37 °C overnight, chilled and diluted with PBS to 40 colony forming units (c.f.u.)/ml for mammary gland infusions. After the infusions an aliquot of each preparation was replated to confirm bacteria count. Mean concentration was found to be 80 ± 3 c.f.u. in the 2-ml infusion. The three strains of *S. aureus* used were chosen because together they account for 100% of the US and 98% of the European *S. aureus* mastitis-causing serotypes[48].

***S. aureus* mammary gland challenge.** Forty-eight hours before initiating the bacterial challenges, health of the animals was assessed by differential leukocyte and milk somatic cell counts to verify that they were within normal ranges. Milk from each quarter, cultured overnight also had to be free of bacterial growth for the animal to be included in the study. Since the udder contains four glands that function independently of one another, it is possible to expose a cow to multiple treatments, as was done in these experiments.

After the morning milking, an aseptic milk sample was collected from each of the four glands before infusing 2 ml of three different strains of *S. aureus* (one per gland) via the streak canal. The fourth gland received 2 ml of sterile PBS. Treatments were assigned such that no gland received the same strain in subsequent trials. The cows were closely monitored, initially at 6-h intervals, and then every 12 h for 48 h. Body temperature, blood and milk samples were taken at 12-h intervals or more frequently throughout the study. Milk samples (20 μl) were plated on blood agar and incubated at 37 °C for 18 to 24 h. Once an infection was confirmed by the presence of viable *S. aureus* in two consecutive milk samples, all four quarters were treated with 10 ml of Pirsue (pirlimycin hydrochloride, Pfizer) for five consecutive milkings. Milk somatic cells and bacteria were monitored weekly thereafter to assure that infections were eliminated.

For enumeration of somatic cells, milk samples were heated to 60 °C for 15 min[49], cooled to 40 °C and cells counted on a Fossomatic 90 (Foss Electric). The device was calibrated quarterly with bovine milk somatic cell standards (Dairy Quality Control Institute Services). Samples were counted in duplicate.

Lactation induction. Prepubertal heifers 4 to 5 months old and weighing 90 to 150 kg were induced to lactate as previously described[50]. Bulls 3 to 6 months of age, weighing between 80 and 160 kg, were induced to lactate by a slightly modified protocol. Briefly, bulls were given twice daily intramuscular injections of estradiol-17β (0.1 mg/kg body weight, Steraloids) and progesterone (0.25 mg/kg, Sigma) for 14 d followed by intramuscular injections of reserpine (2.5 mg/day, Prescription Specialties) on days 15–17. Dexamethasone (20 mg/day, Butler) was administered intramuscularly on days 18–21 and bulls were hand milked for the next 5 d.

Milk characterization. Milk volume, fat and protein were determined from monthly sampling under the supervision of a representative of the Dairy Herd Improvement Association. Lysostaphin and β-lactoglobulin milk concentrations were determined by ELISA as previously reported[23], or as per manufacturer's directions (Bethyl Laboratories), respectively. Milk samples were prepared for ELISA by addition of an equal volume of PBS containing 50 mM EDTA, followed by centrifugation (13,000*g*, 10 min) and collection of milk infranatant (middle fraction) for analysis. Samples were assayed in duplicate. Interassay coefficient of variation was 16.5%. Milk protein profiles by gel electrophoresis and western blot analysis of milk lysostaphin were done as previously described[23].

Milk sample collection. Milk was either collected from an automated sampling device during the normal process of milking or collected by hand in an aseptic manner. All samples were centrifuged at 800*g* for 15 min at 4 °C and the infranatant was collected and tested immediately or frozen at −20 °C until use. Milk samples used in tests involving bacterial growth were first pasteurized by heating to 63 °C for 30 min.

Acute-phase protein assays. To determine whether intramammary challenge with *S. aureus* could elicit an acute-phase response in both the transgenic and nontransgenic cows, plasma samples were collected at varying times before and after challenge, and circulating levels of SAA and LBP were measured by ELISA, as previously described[29,30].

***In vitro* milk assays.** Spot-on-lawn assays were performed to compare the lytic activity of milk from transgenic cows with that of recombinant lysostaphin[51]. Samples were assayed in triplicate and a dilution series of recombinant lysostaphin was always included in the assay. Six hundred microliters of *S. aureus* capsular serotype 5 in log-phase growth containing $\sim 10^5$ c.f.u. were added to 150-mm sterile square Petri dishes containing 12 ml of tryptic soy agar (TSA). Dishes were tilted to distribute the cells uniformly. Dishes were air dried for 30–60 min before 10 µl of a dilutions series (from undiluted to 1:32) of pasteurized test milk samples or recombinant lysostaphin standards, both diluted in skim milk (Difco Laboratories), were added to the dishes. After an additional 30-min drying time, dishes were inverted and incubated at 37 °C in air overnight. Optical density of the cleared zones was determined with a ChemiDoc XRS with Quantity One software (Bio-Rad Laboratories). In a similar manner, lawns of a variety of other bacteria including *E. coli, K. pneumoniae, P. aeruginosa, S. marcescens, S. agalactiae, S. dysgalactiae, S. uberis* and *Staphylococcus hyicus,* were also subjected to transgenic milk and recombinant lysostaphin. Interassay coefficient of variation was 37%.

Statistical methods. The general linear model of univariate and multivariate analysis of variance was used to determine probability values for differences in milk parameters, body temperature and acute-phase serum proteins (SPSS for Windows, version 11, SPSS, Inc.). Percentages, such as percentage milk fat, were first arcsin transformed before ANOVA analysis. Main effect of genotype (transgenic or not) was always included in the model. Other effects such as stage of lactation, breed and *S. aureus* serotype were included in the model when appropriate. All two-way interactions were evaluated. Least square means and their standard errors are reported throughout. Infection rate data were analyzed by chi-square analysis. Densitometry results from the spot-on-lawn assay were analyzed using a nonlinear regression curve fitting function of SigmaPlot (SigmaPlot for Windows version 8.0, Systat Software). A hyperbolic decay curve in the form of $f = y_0 + ae^{(-bx)}$ was found to fit the data best ($r^2 > 0.97$).

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The authors declare competing financial interests; see the *Nature Biotechnology* website for details.

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1. Hammer, R.E. *et al.* Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**, 680–683 (1985).
2. Krimpenfort, P. *et al.* Generation of transgenic dairy cattle using *in vitro* embryo production. *Bio/Technology* **9**, 844–847 (1991).
3. Wright, G. *et al.* High level expression of active human α-1-antitrypsin in the milk of transgenic sheep. *Bio/Technology* **9**, 830–834 (1991).
4. Velander, W.H. *et al.* High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. *Proc. Natl. Acad. Sci. USA* **89**, 12003–12007 (1992).
5. Ebert, K.M. *et al.* Transgenic production of a variant of human tissue-type plasminogen activator in goat milk: generation of transgenic goats and analysis of expression. *Bio/Technology* **9**, 835–838 (1991).
6. Swanson, M.E. *et al.* Production of functional human hemoglobin in transgenic swine. *Bio/Technology* **10**, 557–559 (1992).
7. Fodor, W.L. *et al.* Expression of a functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogeneic hyperacute organ rejection. *Proc. Natl. Acad. Sci. USA* **91**, 11153–11157 (1994).
8. Bleck, G.T., White, B.R., Miller, D.J. & Wheeler, M.B. Production of bovine α-lactalbumin in the milk of transgenic pigs. *J. Anim. Sci.* **76**, 3072–3078 (1998).
9. Brophy, B. *et al.* Cloned transgenic cattle produce milk with higher levels of β-casein and κ-casein. *Nat. Biotechnol.* **21**, 157–162 (2003).
10. Pursel, V.G. *et al.* Growth and tissue accretion rates of swine expressing an insulin-like growth factor 1 transgene. *Anim. Biotechnol.* **15**, 33–45 (2004).
11. Reh, W.A. *et al.* Hot topic: using a stearoyl-CoA desaturase transgene to alter milk fatty acid composition. *J. Dairy Sci.* **87**, 3510–3514 (2004).
12. Saeki, K. *et al.* Functional expression of a Delta12 fatty acid desaturase gene from spinach in transgenic pigs. *Proc. Natl. Acad. Sci. USA* **101**, 6361–6366 (2004).
13. Crittenden, L.B. & Salter, D.W. Gene insertion: current progress and long-term goals. *Avian Dis.* **30**, 43–46 (1986).
14. Clements, J.E. *et al.* Development of transgenic sheep that express the visna virus envelope gene. *Virology* **200**, 370–380 (1994).
15. Sordillo, L.M. & Streicher, K.L. Mammary gland immunity and mastitis susceptibility. *J. Mammary Gland Biol. Neoplasia* **7**, 135–146 (2002).
16. Jasper, D.E. *et al.* Bovine mastitis research. Needs, funding and sources of support. in *Proceedings of the 21st Annual Meeting of the National Mastitis Council*, 182–193, (National Mastitis Council, Washington, DC, 1982).
17. Esslemont, D. & Kossaibati, M. Mastitis: how to get out of the dark ages. *Vet. J.* **164**, 85–86 (2002).
18. Grohn, Y.T., Eicker, S.W., Ducrocq, V. & Hertl, J.A. Effect of diseases on the culling of Holstein dairy cows in New York State. *J. Dairy Sci.* **81**, 966–978 (1998).
19. Sanchez, M.S., Ford, C.W. & Yancey, R.J., Jr. Effect of tumor necrosis factor-α, interleukin-1β, and antibiotics on the killing of intracellular *Staphylococcus aureus*. *J. Dairy Sci.* **77**, 1251–1258 (1994).
20. Schindler, C.A. & Schuhardt, V.T. Lysostaphin: a new bacteriolytic agent for the Staphylococcus. *Proc. Natl. Acad. Sci. USA* **51**, 414–421 (1964).
21. Schuhardt, V.T. & Schindler, C.A. Lysostaphin therapy in mice infected with *Staphylococcus aureus*. *J. Bacteriol.* **88**, 815–816 (1964).
22. Bramley, A.J. & Foster, R. Effects of lysostaphin on *Staphylococcus aureus* infections of the mouse mammary gland. *Res. Vet. Sci.* **49**, 120–121 (1990).
23. Kerr, D.E. *et al.* Lysostaphin expression in mammary glands confers protection against staphylococcal infection in transgenic mice. *Nat. Biotechnol.* **19**, 66–70 (2001).
24. Powell, A.M. *et al.* Cell donor influences success of producing cattle by somatic cell nuclear transfer. *Biol. Reprod.* **71**, 210–216 (2004).
25. Ebert, K.M. *et al.* Induction of human tissue plasminogen activator in the mammary gland of transgenic goats. *Bio/Technology* **12**, 699–702 (1994).
26. Cammuso, C. *et al.* Hormonal induced lactation in transgenic goats. *Anim. Biotechnol.* **11**, 1–17 (2000).
27. Heddaeus, H., Heczko, P.G. & Pulverer, G. Evaluation of the lysostaphin-susceptibility test for the classification of staphylococci. *J. Med. Microbiol.* **12**, 9–15 (1979).
28. Grinde, B. A lysozyme isolated from rainbow trout acts on mastitis pathogens. *FEMS Microbiol. Lett.* **51**, 179–182 (1989).
29. Bannerman, D.D. *et al. Escherichia coli* and *Staphylococcus aureus* elicit differential innate immune responses following intramammary infection. *Clin. Diagn. Lab. Immunol.* **11**, 463–472 (2004).
30. Gronlund, U., Hulten, C., Eckersall, P.D., Hogarth, C. & Waller, K.P. Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced *Staphylococcus aureus* mastitis. *J. Dairy Res.* **70**, 379–386 (2003).
31. Lund, T., Miglior, F., Dekkers, J.C.M. & Burnside, E.B. Genetic relationship between clinical mastitis, somatic cell count, and udder conformation in Danish Holsteins. *Livest. Prod. Sci.* **39**, 243–251 (1996).
32. McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J. & Hennighausen, L. Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc. Natl. Acad. Sci. USA* **89**, 6943–6947 (1992).
33. Artelt, P. *et al.* The prokaryotic neomycin-resistance-encoding gene acts as a transcriptional silencer in eukaryotic cells. *Gene* **99**, 249–254 (1991).
34. Schukken, Y.H. *et al.* Experimental *Staphylococcus aureus* intramammary challenge in late lactation dairy cows: quarter and cow effects determining the probability of infection. *J. Dairy Sci.* **82**, 2393–2401 (1999).
35. Paape, M.J., Schultze, W.D., Guidry, A.J., Kortum, W.M. & Weinland, B.T. Effect of intramammary polyethylene device on the concentration of leukocytes and immunoglobulins in milk and on the leukocyte response to *Escherichia coli* endotoxin and challenge exposure with *Staphylococcus aureus*. *Am. J. Vet. Res.* **42**, 774–783 (1981).
36. Uhlar, C.M. & Whitehead, A.S. Serum amyloid A, the major vertebrate acute-phase reactant. *Eur. J. Biochem.* **265**, 501–523 (1999).
37. Schumann, R.R. & Latz, E. Lipopolysaccharide-binding protein. *Chem. Immunol.* **74**, 42–60 (2000).
38. Schroder, N.W. *et al.* Lipoteichoic acid (LTA) of *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-like receptor (TLR-2, lipopoly-

saccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *J. Biol. Chem.* **278**, 15587–15594 (2003).
39. Salter, D.W. *et al.* Gene insertion into the chicken germ line by retroviruses. *Poult. Sci* **65**, 1445–1458 (1986).
40. Van Berkel, P.H. *et al.* Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nat. Biotechnol.* **20**, 484–487 (2002).
41. Maga, E.A., Anderson, G.B., Cullor, J.S., Smith, W. & Murray, J.D. Antimicrobial properties of human lysozyme transgenic mouse milk. *J. Food Prot.* **61**, 52–56 (1998).
42. Mitra, A. *et al.* Expression of lysostaphin in milk of transgenic mice affects the growth of neonates. *Transgenic Res.* **12**, 597–605 (2003).
43. Harrison, E.F., Fuquay, M.E. & Zygmunt, W.A. Antigenic response to topically applied proteins. *Infect. Immun.* **11**, 309–312 (1975).
44. Harris, R.L., Nunnery, A.W. & Riley, H.D., Jr. Effect of lysostaphin on staphylococcal carriage in infants and children. *Antimicrob. Agents Chemother.* **1967**, 110–112 (1968).
45. Martin, R.R. & White, A. The selective activity of lysostaphin *in vivo*. *J. Lab. Clin. Med.* **70**, 1–8 (1967).
46. Dajcs, J.J. *et al.* Lysostaphin is effective in treating methicillin-resistant *Staphylococcus aureus* endophthalmitis in the rabbit. *Curr. Eye Res.* **22**, 451–457 (2001).
47. Cavadini, C., Hertel, C. & Hammes, W.P. Application of lysostaphin-producing lactobacilli to control staphylococcal food poisoning in meat products. *J. Food Prot.* **61**, 419–424 (1998).
48. Guidry, A. *et al.* Serotyping scheme for *Staphylococcus aureus* isolated from cows with mastitis. *Am. J. Vet. Res.* **59**, 1537–1539 (1998).
49. Miller, R.H., Paape, M.J. & Acton, J.C. Comparison of milk somatic cell counts by Coulter and Fossomatic Counters. *J. Dairy Sci.* **69**, 1942–1946 (1986).
50. Ball, S., Polson, K., Emeny, J., Eyestone, W. & Akers, R.M. Induced lactation in prepubertal Holstein heifers. *J. Dairy Sci.* **83**, 2459–2463 (2000).
51. Barry, A.L. Procedure for testing antibiotics in agar media: theoretical considerations. in *Antibiotics in Laboratory Medicine* (ed. Lorian, V.) 1–23 (Williams and Wilkins, Baltimore, 1980).

Corrigendum: Genetically enhanced cows resist intramammary *Staphylococcus aureus* infection

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In the abstract on page 445, the quantities given for lysostaphin in the following sentences are incorrect: "To test the feasibility of protecting animals through genetic engineering, transgenic cows secreting lysostaphin at concentrations ranging from 0.9 to 14 mg/ml in their milk were produced" and "Protection against *S. aureus* mastitis appears to be achievable with as little as 3 mg/ml of lysostaphin in milk." The correct quantities are 0.9 to 14 μg/ml and 3 μg/ml, respectively. The quantities are correct in the rest of the paper.

Corrigendum: Transient inhibition of BMP signaling by Noggin induces cardiomyocyte differentiation of mouse embryonic stem cells

Shinsuke Yuasa, Yuji Itabashi, Uichi Koshimizu, Tomofumi Tanaka, Keijiro Sugimura, Masayoshi Kinoshita, Fumiyuki Hattori, Shin-ichi Fukami, Takuya Shimazaki, Hideyuki Okano, Satoshi Ogawa & Keiichi Fukuda

Nat. Biotechnol. **23**, 607–611 (2005)

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